# Context-dependent memory traces in the crab's mushroom bodies: Functional support for a common origin of high-order memory centers

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The hypothesis of a common origin for the high-order memory centers in bilateral animals is based on the evidence that several key features, including gene expression and neuronal network patterns, are shared across several phyla. Central to this hypothesis is the assumption that the arthropods' higher order neuropils of the forebrain [the mushroom bodies (MBs) of insects and the hemiellipsoid bodies (HBs) of crustaceans] are homologous structures. However, even though involvement in memory processes has been repeatedly demonstrated for the MBs, direct proof of such a role in HBs is lacking. Here, through neuroanatomical and immunohistochemical analysis, we identified, in the crab *Neohelice granulata*, HBs that resemble the calyxless MBs found in several insects. Using in vivo calcium imaging, we revealed training-dependent changes in neuronal responses of vertical and medial lobes of the HBs. These changes were stimulus-specific, and, like in the hippocampus and MBs, the changes reflected the context attribute of the memory trace, which has been envisioned as an essential feature for the HBs. The present study constitutes functional evidence in favor of a role for the HBs in memory processes, and provides key physiological evidence supporting a common origin of the arthropods' high-order memory centers.

memory centers | evolution | homology | Arthropoda | in vivo Ca$^{2+}$ imaging

Learning skills vary across species depending upon specific adaptations to environmental features (1). However, beyond such adaptations, different species share many of the basic mechanisms involved in learning and memory. Both the molecular machinery involved in neural plasticity and the dynamics of the memory processes are conserved throughout evolution (2–5). This characteristic is critical to the hypothesis of a common origin of the high-order memory centers in bilateral animals (6, 7), centers that play a fundamental role in learning and memory by orchestrating high-order sensory processing within contextual frameworks (8, 9). The idea that these centers evolved from the same structure in a common ancestor has been reborn after the remarkable study of Tomer et al. (7) indicating deep homology of mushroom bodies (MBs) and the vertebrate pallium that dates back the origin of higher brain centers to the protostome-deuterostome ancestor times. The vertebrate pallium and the annelid MBs have a conserved overall molecular brain topology and neuron types (7). Furthermore, MBs and the hippocampus' dentate gyrus share the ability to generate new neurons during adult life (6, 10, 11). In this context, a recent study by Wolff and Strausfeld (6) has proposed that the similarities in both neuronal architectures and protein expression patterns between the mammalian hippocampus, the MBs, and the hemiellipsoid bodies (HBs) of crustaceans are important indicators of genealogical correspondence.

MBs are complex paired structures of the forebrain of invertebrate species and have been vastly studied in insects (12). Since their description in the mid-1850s, the MBs have been considered higher order brain centers involved in memory processes, "compared them with the convolutions of the human brain, and even thought them associated with hexapod intelligence" (13). Although a number of studies show that these centers have olfaction-based functions, MBs are also involved in several behavioral functions (14). Accordingly, insect MBs have been described as one of the key neural structures to encode and retain experiences not just in olfactory instances but also in spatial- and context-dependent memory paradigms (3, 8, 14–17). As for the hippocampus in the vertebrate brain (9), the MBs have been proposed to be involved in linking learned items within a contextual framework (8).

Supporting the hypothesis of a common origin for the high-order memory centers in bilateral animals are the studies showing evidence of structural homology between the MBs of insects and the HBs of crustaceans (12, 18). The similarities between MBs and HBs in regard to morphological and immunoreactive patterns [including the major catalytic subunit of protein kinase A and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII), proteins required in memory processes] are relevant evidence of genealogical correspondence of these arthropod brains' higher centers (12, 19). Moreover, HBs also generate new neurons during adult life (20, 21). The concordance among several types of data, including topology, topography, gene expression, and functions, are useful to support phylogenic and/or structural homologies in the central nervous system (6, 22–24). Based on solid evidence about

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**Significance**

Mushroom bodies (MBs) are higher brain structures of several invertebrate groups, vastly studied in insects as a key structure involved in memory processes. Moreover, MBs and the vertebrate pallium have been proposed to share an ancestral common origin. In crustaceans, the hemiellipsoid bodies (HBs) are proposed to be homologues of the insect MBs. However, functional evidence for the involvement of HBs in memory processes is lacking. Here, in the crab *Neohelice*, we show memory traces in the HBs that, as for MBs, reflect the context attribute of memory. These results extend the homology based on anatomy and gene expression to the functional level. Consequently, present data support the hypothesis of a common origin for the arthropods' high-order memory centers.

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the MB function, the HB has been largely predicted as a high-order memory center (6, 25, 26). However, to the extent of our knowledge, there is yet no direct evidence in favor of the occurrence of learning and memory processes in this neuropil. Here, we evaluated whether, as expected for high-order memory centers (17, 27), the HB's activity reflects the link between a learned item and the context with which it has been associated.

In the memory paradigm developed in the crab *Neohelice* (*Chasmagnathus*) *granulata* (Fig. 1*A*), animals learn to associate the training context with a visual danger stimulus (VDS) passing overhead (28, 29). Several neuronal correlates of memory processes in this paradigm have been studied using biochemical, molecular, and electrophysiological approaches (29). A group of motion-sensitive neurons that project from the lobula (Fig. 1*B*) to the lateral protocerebrum reflects short- and long-term behavioral changes related to the VDS but not to the context specificity of the memory (29, 30). A high-order memory trace linking the VDS with the training context was envisioned to reside in the HBs (30).

In the present study, we combine histological and immunohistochemical analyses, neuronal recordings, and behavioral experiments to examine putative neuronal correlates of memory in the HBs of the crab *Neohelice*. Neuroanatomical and immunohistochemical data revealed HBs whose intrinsic neurons [globuli cells (gc) in a cluster showing adult neurogenesis] form a parallel-projecting bundle of neurites (pedunculus) that then divide to outline two main lobes, resembling the anatomy of calyxless MBs observed in some insect species. In vivo calcium imaging showed that the neural activity of HBs, elicited by the training stimulus, is modified by training. Moreover, the data revealed a context-specific memory trace in the HBs.

## Results

**HBs of the Crab *N. granulata*, Histology, and Immunostaining.** In decapod crustaceans, the brain is formed by the proto-, deuto-, and tritocerebrum. The optic lobes, located within eyestalks and connected with the rest of the brain via the protocerebral tract, are part of the protocerebrum and comprise the lamina, medulla, lobula, lobula plate, and lateral protocerebrum (Fig. 1*B*). The lateral protocerebrum comprises, in turn, several discrete neuropils, including the medulla terminalis and the HB. The medulla terminalis is a generic name used for several closely packed neuropils that receive input from the olfactory globular tract, the ipsilateral and contralateral optic neuropils, and the HB (25, 31). In *Neohelice*, as in other crabs, a marked division between the HB and the medulla terminalis is not obvious (31–34). In the present study, the nomenclature proposed for the HB is based on the nomenclature used for calyxless MBs of insects (15).

The intrinsic components of the HB, as for the MB, derive from a cluster of minute basophilic gc (12, 15). Golgi impregnations, in which the HB-gc were revealed (Fig. 1*C*), show that the basic structure of the HB in *Neohelice* resembles the organization of the calyxless MB of insects: a cluster of small cell somata projecting parallel fibers constituting the pedunculus, which bifurcates twice. The first bifurcation, oriented dorsally, originates what we called the vertical lobe [in analogy to the insect's MB (15); Fig. 1*D*]. The second bifurcation, oriented anterior-laterally, originates the medial lobes (constituted by two close structures, medial lobe I and medial lobe II), called neuropils I and II in crayfish and other Decapoda (26, 31). Fig. 1*E* shows a magnification where particular gc fibers can be seen bifurcating and ramifying in all of the lobes. In close parallel to Kenyon cells of insect MBs and gc from HBs of hermit crabs, intrinsic cells of the HBs in *Neohelice* show specializations similar to claw terminals (19, 35) (Fig. 1*F*). One possible input to the HB could be the bistratified lobula giant 2 (BLG2) neurons. Fig. 1*G* shows an intracellularly stained BLG2 that has processes entering the two HB medial lobes. Interestingly, the BLG2 neurons respond to visual and mechanical stimulation (36).
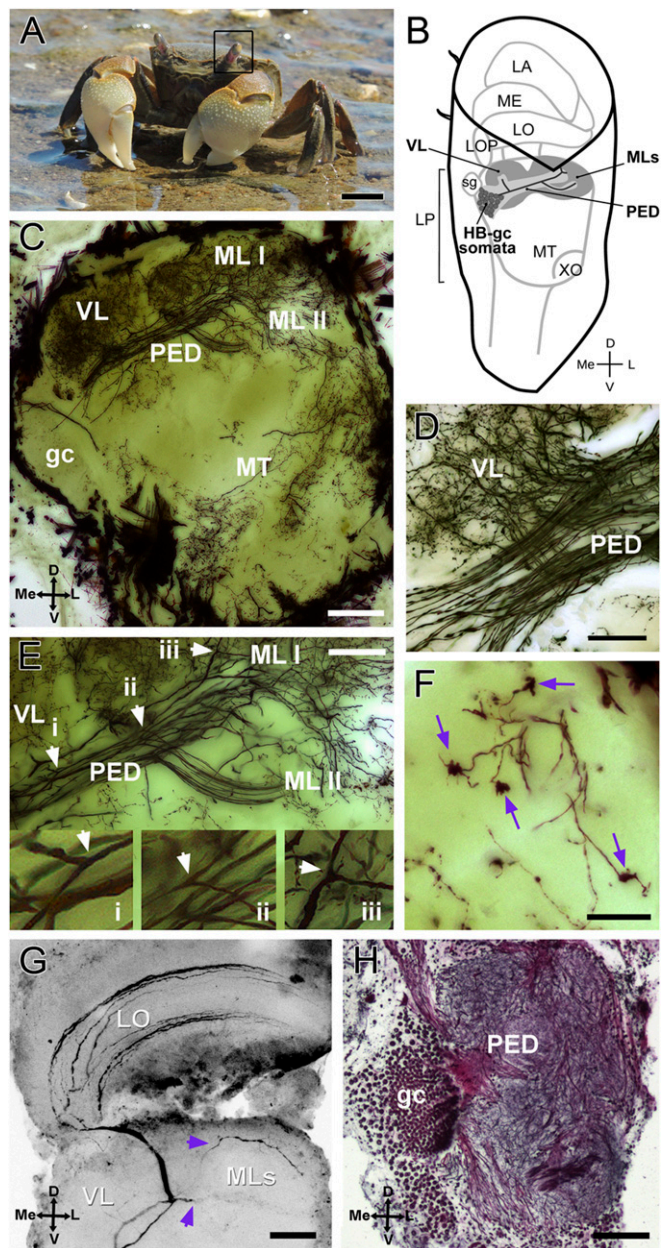


**Fig. 1.** Crab *N. granulata* and the HB. (*A*) *N. granulata* crab. The black frame surrounds the left eyestalk. (*B*) Scheme showing the position and shape of the neuropils within the eyestalk. The HB is part of the lateral protocerebrum and is drawn in gray. Complete gc and some somata of the HB are drawn. (*C–F*) Golgi impregnation of the optic lobes (50-μm sections). (*C*) Overall structure of the HB seems mainly formed by intrinsic cells derived from the HB-gc cluster. (*D*) Detail of the bifurcation site that originates the vertical lobe. (*E*) Detail of the bifurcation sites that give rise to the vertical lobe and to both medial lobes. (*Insets*) Arrowheads show three amplified bifurcations (*i*, *ii*, and *iii*) in the same neuron. (*F*) Claw-like specializations are present in the medial lobes (arrows). (*G*) Intracellularly stained BLG2 neuron showing two branches that invade the two medial lobes of the HB (arrowheads). (*H*) Bodian-stained section (12 μm) of the lateral protocerebrum showing gc and the pedunculus. D, dorsal; L, lateral; LA, lamina; LO, lobula; LOP, lobula plate; LP, lateral protocerebrum; Me, medial; ME, medulla; MT, medulla terminalis; PED, pedunculus; MLs, medial lobes; sg, sinus gland; V, ventral; VL, vertical lobe; XO, X-organ. (Scale bars: *A*, 1 cm; *C*, *G*, and *H*, 100 μm; *D* and *E*, 50 μm; *F*, 25 μm.)

Intrinsic neurons (HB-gc) constituting the HB are numerous, near a thousand (Movies S1 and S2). Part of the HB-gc somata cluster and the fibers forming the pedunculus are shown in a

Bodian-stained section of the lateral protocerebrum (Fig. 1*H*). HB-gc somata are easily distinguished from other surrounding somata by their size, smaller than neighboring cells.

Synapsins (SYNs), proteins associated with synaptic vesicles, are effective for revealing synaptic neuropils in arthropods because they are abundantly expressed in regions of high synaptic density. Fig. 2*A* shows a confocal section of the lateral protocerebrum for whole-mount immunohistochemistry against SYN. We found strong SYN immunoreactivity in regions that correspond to the arborization areas of the vertical and medial lobes of the HB, but not in the pedunculus. Both vertical and medial lobes show a SYN immunoreactivity texture consistent with a microglomeruli structure as described in HBs of other crustacean and in MBs (19, 31, 35). Fig. 2 *B* and *C* show details obtained by digital zoom of areas of the vertical (Fig. 2*B*) and medial (Fig. 2*C*) lobes, where traits compatible with microglomeruli structures can be recognized as rounded areas delimited by high SYN immunoreactivity.

As in other crustaceans, we found a strong allatostatin-like immunoreactivity in the closely related medulla terminalis neuropils, but not in the HB (37) (Fig. 2*D*). Adult MB neurogenesis has been previously observed in some insect species (38) and in the HB somata cluster of other crustaceans (20, 21). In vivo 5-bromo-2′-deoxyuridine (BrdU) labeling stained HB-gc somata, suggesting neurogenesis in a zone of the somata cluster proximal to the neck of the pedunculus (Fig. 2*E*). The expression of proteins associated with mechanisms of memory processes, like p-CaMKII-α, is another characteristic of forebrain centers such as MBs and HBs (12). The p-CaMKII-α immunoreactivity reveals the medial and vertical lobes (Fig. 2*F*). Moreover, HB-gc somata show strong p-CaMKII-α immunoreactivity, in contrast to their bigger neighboring cells.

## Memory-Related Neural Plasticity in the Crab's HBs.

The memory paradigm used in *Neohelice* is based on the crab's escape response elicited by a VDS resembling an aerial predator. Upon repeated presentations (15 spaced trials) of the VDS, the escape response declines over trials turning into a freezing behavior. The change in behavior persists in the long term, and is mediated by an association between a training context (a bowl that receives above-light illumination) and VDS (29, 39–41). The training protocol regularly used in *Neohelice* yields a decrease in escape response that, in the short-term, is not specific to the training context (42). However, the goal of this study is to evaluate whether the activity of HBs might reflect context specificity during imaging experiments, which are tested in the short term. Hence, in the present study, we switched to a slightly different version of the training protocol that enhances the associative strength between VDS and context (43). Here, we tested whether this protocol yields short-term memory that, like long-term memory, is context-specific. Fig. 3 *A*, *i* shows a scheme of the experimental procedure. The experimental device, the actometer (28, 29), consisted of a bowl-shaped opaque container where the crab was lodged during training and testing sessions. During each trial, a VDS (an opaque rectangular screen) was moved twice horizontally in a 90° clockwise and counterclockwise excursion from the starting position and back. The VDS induces an escape response in the crab, and consequent container vibrations are converted into electrical signals through microphones placed on the external wall of the container. The activity of the crab was recorded throughout each VDS presentation (9 s) in arbitrary units. The actometer was illuminated from above during the training trials and from below during intertrial intervals (153 s) (Fig. 3 *A*, *i*).

The experiment consisted of two pairs of groups, with each pair comprising an untrained group (UN) and a trained group
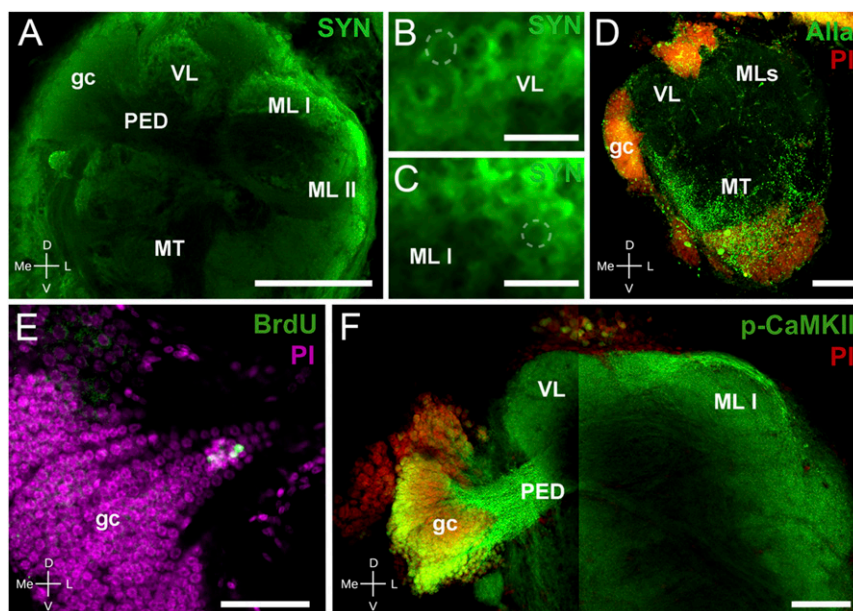


**Fig. 2.** Immunohistochemistry and neurogenesis in *Neohelice*'s HBs. (*A–C*) Confocal laser scan of the lateral protocerebrum labeled with antibodies against SYN (green). (*A*) The gc somata cluster, the pedunculus, and both the vertical and medial lobes (medial lobes I and II) composing the HB can be distinguished. (*B*) Detail of the vertical lobe showing traits resembling microglomeruli (dashed line points out an example). (*C*) Same as *B* for the medial lobe I. (*D*) Maximum projection of confocal laser scan sections showing immunoreactivity against allatostatin (Alla, green) and nuclei stained with propidium iodide (PI, red). Alla-like immunoreactivity appears widely distributed in the medulla terminalis, whereas there is scarce immunoreaction in the area corresponding to the HB. (*E*) Confocal laser scan sections of the HB-gc somata labeled with antibody against BrdU (green) and with PI (magenta) visualizing newborn cells in the gc cluster. (*F*) Confocal laser scan of the HB labeled with antibodies against p-CAMKII-α (green) and with PI (red). The image is composed of two stacks taken at different focal planes. (*Left*) The gc, comarked p-CAMKII-α and PI, and the pedunculus that projects toward the vertical and medial lobes. (*Right*) Pedunculus projecting toward both medial lobes. Abbreviations are as in Fig. 1. (Scale bars: *A*, 200 μm; *B* and *C*, 20 μm; *D* and *F*, 100 μm; *E*, 50 μm.)
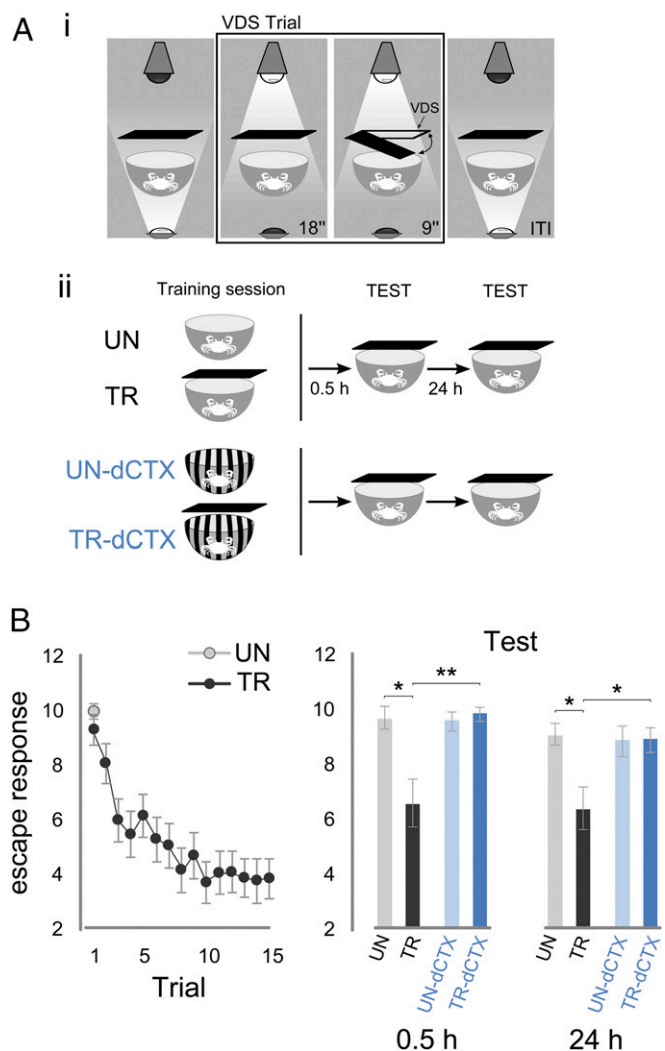
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**Fig. 3.** Behavioral experiments and memory in the crab *Neohelice*. (*A, i*) Scheme of a VDS trial. A crab is inside the actometer that is illuminated from below during the intertrial interval (ITI) for 153 s. During the VDS trial, the actometer is illuminated from above for 27 s. During the last 9 s, the VDS moves twice in a 90° clockwise and counterclockwise excursion from the starting position. (*A, ii*) Scheme showing the different groups of crabs. The TR receives 15 VDS trials, whereas the UN receives the VDS once, only in the first of the 15 trials. UN-dCTX and TR-dCTX are trained with the same protocol as UN-TR but in a different context (actometers with striped walls). In the TEST sessions, 0.5 and 24 h later, all four groups receive a VDS trial in the actometer without stripes. Thus, UN-TR crabs are tested in the same context in which they were trained, whereas UN-TR-dCTX crabs are tested in a different context from training. (*B, Left*) Escape response (log$_2$-transformed) of crabs to the VDS throughout the 15 spaced trials of the training session. (*B, Right*) During the memory retention tests 0.5 and 24 h later, crabs exhibited memory when tested in the same training context (TR or UN), but not when the testing context was different from the one at training (TR-dCTX or UN-dCTX). For each group, $n = 28$. Games–Howell post hoc test: *$P < 0.05$, **$P < 0.01$. Plots show mean escape response (log$_2$-transformed) ± SEM.

(TR) of animals. During training session, the TR received 15 VDS trials, whereas the UN received only one VDS trial. Two test sessions were run with the same animals: short term at 0.5 h and long term at 24 h after training. To evaluate the memory's context specificity, the UN-TR pair was trained and tested in the same context and the UN-TR tested in a different context (dCTX) pair was tested in a context different from the one used during training (Fig. 3 *A, ii*). No difference was found for the first training trial between TRs and UNs (Welch's *t* test: UN vs. TR:

$t[39.89] = 1.04$, $P = 0.30$, $n = 28,28$), and a significant trial effect was evidenced for the TR during training (Fig. 3*B*, *Left*) (repeated measures ANOVA. TR: $F[7.60,205.31] = 9.55$, $P < 0.001$, epsilon = 0.54, $n = 28$; Greenhouse–Geisser correction for sphericity violation). During the short-term memory test session (0.5 h), crabs expressed memory when the test was run in the training context (UN vs. TR) but not when the test was run in a different context from the one used during training (UN-dCTX vs. TR-dCTX) (Fig. 3*B*, *Right*) (one-way ANOVA Welch's test: $F[3, 57.23] = 4.13$, $P = 0.01$; post hoc Games–Howell test: UN-TR: $q[38.76] = 4.53$, $P < 0.016$; UN-dCTX–TR-dCTX: $q[49.71] = -0.86$, $P > 0.05$; TR–TR-dCTX: $q[31.62] = -5.02$, $P < 0.007$; UN–UN-dCTX: $q[48.84] = 0.54$, $P > 0.05$). As expected (43), long-term memory (24 h) evaluated in the same groups of animals showed similar results (Fig. 3*B*, *Right*) (one-way ANOVA Welch's test: $F[3, 58.76] = 3.27$, $P = 0.027$; post hoc Games–Howell test: UN-TR: $q[40.27] = -4.38$, $P < 0.0195$; UN-dCTX–TR-dCTX: $q[51.69] = -0.086$, $P > 0.05$; TR–TR-dCTX: $q[43.30] = -3.91$, $P < 0.042$; UN–UN-dCTX: $q[52.23] = 0.39$, $P > 0.05$). Therefore, the use of a training protocol in which a change in the illumination of the training context anticipates the VDS by a few seconds renders context-specific memory expression in the short term as in the long term.

We ran in vivo calcium imaging experiments to examine memory-related changes in neural activity of the crab's HBs. For that aim, a small window was opened in the crab's left eyestalk allowing visual access to the lateral protocerebrum. Small crystals of the calcium-sensitive dye Calcium Green-1 dextran were stabbed into the neck of the pedunculus of the HB. The dye was internalized by gc and transported along the neurons to the HB lobes. Fig. 4 *A, i* shows a diagram of the lateral protocerebrum, where the site of insertion of the dye is shown; a view of the preparation is shown in Fig. 4 *A, ii*. Fig. 4 *A, iii* shows an example of a confocal stack of the lateral protocerebrum incubated with antibodies against SYN. The intense green spot corresponds to the position where Calcium Green was inserted; multiple somata and some processes of HB neurons that were stained with Calcium Green can still be seen after tissue fixation.

During in vivo imaging experiments, calcium signals from the vertical and medial lobes were simultaneously registered while the crab was held in the imaging setup. All recordings were obtained from the HB of the left eyestalk, whereas a training protocol similar to the one described for behavioral experiments was run stimulating the animal through its right eye. It is well known that when the crabs are immobilized in a way that body movements related to escape response are impeded, learning and memory formation still occur (44). A short-term test trial was performed 0.5 h after the end of the training protocol. In addition, a stimulus of a different sensory modality that does not trigger the escape response (45), a mechanical stimulus (Mec), was used at the beginning (MecPRE) and at the end of the imaging experiments (MecPOST) in all experimental groups to control for unspecific changes that may happen to the preparation during the experiment. Fig. 4*B* shows the training protocol (*i*) and a scheme of the timing of the stimuli: VDS (*ii*) and Mec (*iii*) (VDS or Mec is applied twice in each trial). Recordings were made during every Mec, during every VDS, and during nonstimulated trials. Calcium signals were calculated as changes from the basal fluorescence as %$\Delta F/F$ (*SI Materials and Methods*, *In Vivo Optical Imaging Experiments*), where $F$ represents the basal fluorescence and $\Delta F$ represents the difference between the basal fluorescence and the fluorescence measured during the frame of interest.

Both VDS and Mec triggered transient calcium signals in the vertical and medial lobes (Fig. 4*C*). For analysis, calcium responses were quantified as the summation of the %$\Delta F/F$ obtained for each frame during VDS or Mec (Fig. 4*C*, shaded area under the traces). A calcium signal rate (CSR) index (*SI Materials and*
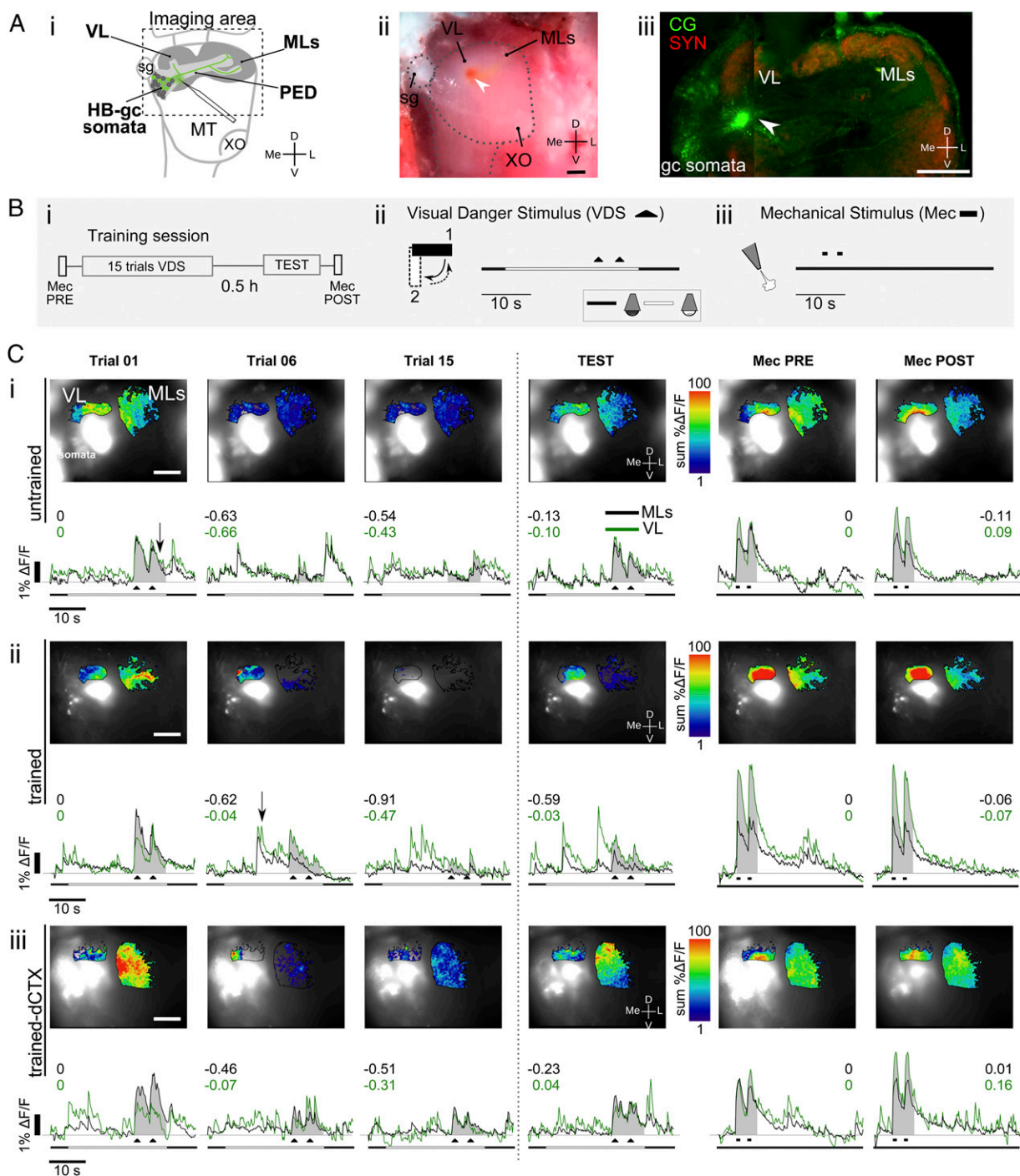
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**Fig. 4.** In vivo calcium imaging preparation and neural activity in the crab's HB. (*A*) Preparation for calcium imaging experiments. (*i*) Diagram of the lateral protocerebrum showing the site where the crystals of calcium dye were stabbed with a glass capillary. A complete gc and some somata of the HB drawn in green represent cells filled with the dye. A dashed frame outlines the approximately recorded area during experiments. (*ii*) Picture of the preparation under the stereomicroscope. The red spot corresponds to the Calcium Green crystals, which look red under white light. (Scale bar: 100 μm.) (*iii*) Confocal stack showing immunoreaction against SYN and Calcium Green staining. (Scale bar: 200 μm.) (*B*) Training protocol and scheme of the stimulation trials. (*i*) Training protocol consists of 15 VDS trials followed by a short-term test 0.5 h later. A Mec is delivered before training (PRE) and after testing (POST) as a control stimulus. (*ii*) VDS consists of a screen moving back and forth 90° above the animal (black triangles). The trial consists of a delayed context-VDS presentation. The time line represents the whole extent of each recording event (40 s), including the 27 s in which illumination changes from below to above, and the VDS toward the end of this period. Dark line, below illumination; white line, above illumination. (*iii*) Mec consists of two 1-s pulses of nitrogen at the dorsal posterior part of the crab's carapace (black rectangle denotes the Mec timing). (*C*) Examples of calcium dynamics. (*Upper*) Superimposed raw and false color-coded images for the ML and VL areas showing neuronal calcium responses as the summation of change in fluorescence (sum %ΔF/F) upon stimulation for training trials 1, 6, and 15 and the test, and Mec for the medial and vertical lobes. The white glow corresponds to intensity saturation, where the dye was stabbed. (Scale bar: 200 μm.) (*Lower*) Time course calcium dynamics as %ΔF/F during trials corresponding to the image in the upper row. The shaded area shows the time window considered for summation of the activity elicited by the stimulus and used for calculation of the CSR. Numbers in black and green show the CSR for each trial and each lobe. MecPRE/POST, Mec pretraining or posttesting. (*i*) UN crab example. (*ii*) TR crab example. (*iii*) TR-dCTX crab example.

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*Methods*, *Statistical Analysis*, *Optical imaging*) was calculated to analyze relative changes in comparison to the first VDS (trial 1) or Mec (MecPRE) trials: CSR = −1 corresponds to a complete reduction of the response, CSR = 0 corresponds to no change, and a positive CSR corresponds to an increase in the response. As for behavioral experiments, four groups were run in the imaging experiments: UN crabs (Fig. 4 *C*, *i*; representative example), TR crabs (Fig. 4 *C*, *ii*; representative example), UN-dCTX crabs, and TR-dCTX crabs (Fig. 4 *C*, *ii*; representative example). It is noteworthy that UNs show negative CSRs in trials 2–15 because they do not receive VDS stimulation along those trials.

Both TRs, TR and TR-dCTX, exhibited responses in the medial and vertical lobes elicited by the VDS. Responses decreased with a similar profile throughout trials and resulted in negative CSRs by the end of the training sessions (Fig. 5 *A* and *B*, *Left*). Moreover, during the last trials of the training sessions, trained animals (TR and TR-dCTX) showed a CSR indistinguishable from the basal activity of unstimulated animals (UN and UN-dCTX) (Fig. S1; block of 13–15 trials, one-way ANOVA: medial lobes: $F[3,21] = 1.01$, $P = 0.40$; vertical lobe: $F[3,21] = 2.64$, $P = 0.076$).

At a short-term test performed 0.5 h after the end of training (Fig. 5*A*; Test), the medial lobes of the HB showed decreased responses to the VDS if the test was run in the training context (UN vs. TR), but not if the test was run in a different context from the one used during training (UN-dCTX vs. TR-dCTX) (one-way ANOVA: $F[3,21] = 18.77$, $P = 0.000004$; Tukey honest significance difference (HSD) test, same context groups: UN vs. TR: $P = 0.00018$, context shift groups: UN-dCTX vs. TR-dCTX: $P = 0.112$, UNs: UN vs. UN-dCTX: $P = 0.99$, TRs: TR vs. TR-dCTX: $P = 0.005$).

In regard to activity measured in the vertical lobe, the short-term test showed a similar profile (Fig. 5*B*; Test) (one-way ANOVA: $F[3,21] = 3.58$, $P = 0.03$). However, the decrease of the VDS-elicited activity in the TR was less pronounced (Tukey

HSD: UN vs. TR: $P = 0.025$, UN-dCTX vs. TR-dCTX: $P = 0.99$, UN vs. UN-dCTX: $P = 0.65$, TR vs. TR-dCTX: $P = 0.29$).

Importantly, the training-dependent change in neuronal responses measured in the HBs was specific to the VDS because responses elicited by the Mec showed no differences in any of the groups before and after the training session (Fig. 5*C*): The CSR was not different from zero in any group, and there were no differences between groups (MecPOST $t$ test single sample: medial lobes and vertical lobes for all groups: $P > 0.32$; one-way ANOVA, medial lobes: $F[3,21] = 0.65$, $P = 0.59$; vertical lobes: $F[3,21] = 0.48$, $P = 0.70$).

In summary, the calcium imaging experiments show changes in neural activity in the crab's HBs in the form of a context stimulus-specific decrease in responses elicited by the VDS that correlates with the crab's memory expression.

## Discussion

Like in other crustaceans, the basic structure of the *Neohelice*'s HBs and the immunoreactive pattern obtained based on p-CaM-KII-α, SYN, and allatostatin resemble the profile of the MBs in insects. Moreover, adult neurogenesis among the intrinsic elements of the HBs further supports this interpretation. Understanding the morphology of *Neohelice*'s HBs allowed us to start uncovering its role in memory processes. Our in vivo calcium imaging results indicate that the HBs undergo stimulus-specific changes during training that last at least until a short-term test 0.5 h after training. Moreover, plasticity might support the context-specific association observed in the behavior.

**HBs in *Neohelice*: Structure and Homology with Insect MBs.** Whether the crustacean HBs are homologous to the insect MBs has been a subject of long debate (25, 31). If demonstrated, the proposed homology would constitute important support in favor of the hypothesis for a common origin of the high-order memory centers in bilateral animals (6, 12). Strong evidence supporting the genealogical
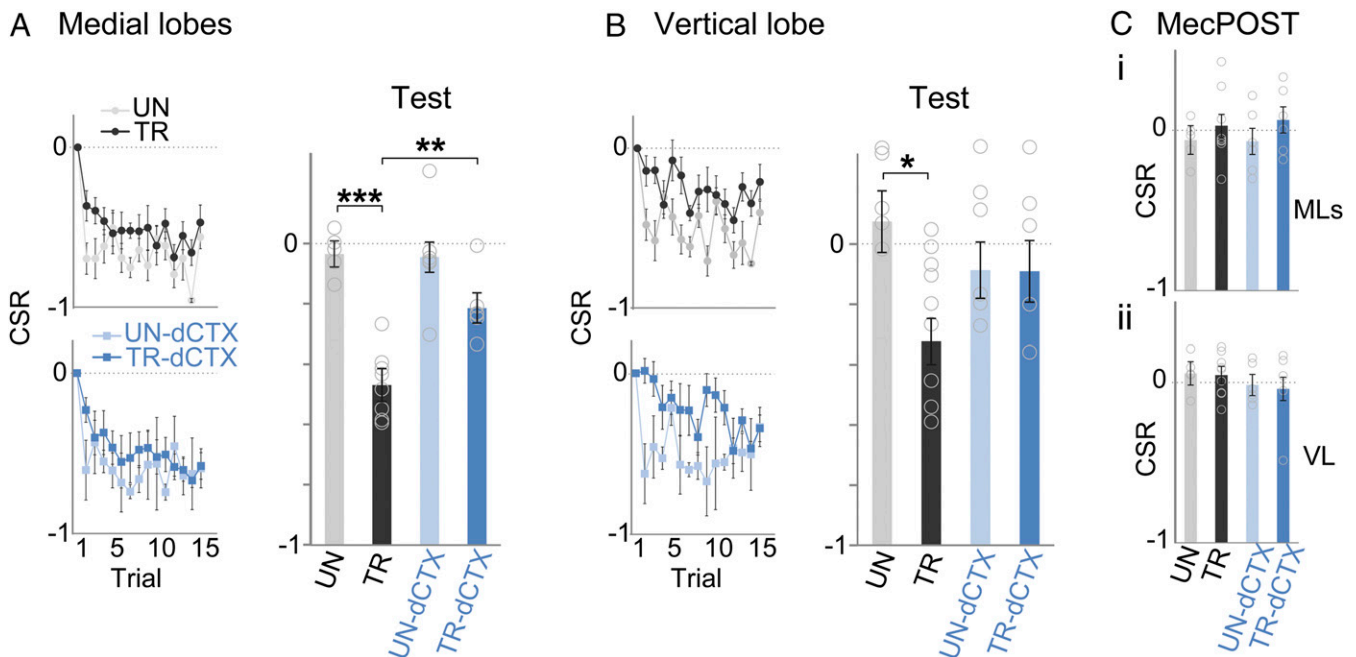


**Fig. 5.** Context-dependent learning plasticity in the HBs. (*A*) HB CSRs elicited with the VDS during training and testing corresponding to the medial lobes (UN, TR, UN-dCTX, and TR-dCTX: $n = 5$, $n = 8$, $n = 6$, and $n = 6$, respectively). (*B*) Same as *A* for the vertical lobe (UN, TR, UN-dCTX, and TR-dCTX: $n = 5$, $n = 9$, $n = 6$, and $n = 5$, respectively). (C) MecPOST. CSRs for medial and vertical lobes are shown. One-way ANOVA, Tukey HSD test: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Circles represent individual animals. CSR = −1 corresponds to a complete reduction of the response, CSR = 0 corresponds to no change, and a positive CSR corresponds to an increase in the response. Plots show mean CSR ± SEM.

correspondence between MBs and HBs is the shared ground pattern of neuronal organization and the similar protein expression profile associated with memory processes that have been described recently (12, 19). The present study provides morphological data and immunoreactivity patterns that reveal the similarity of structures between insect MBs and *Neohelice* HBs.

Bilobed HBs with neuropil regions I and II formed by thousands of microglomeruli were described in crayfish and lobster (31, 32, 46). These lobes were also manifested in the crab by the immunoreactivity against SYN (medial lobe I and medial lobe II; Fig. 2*A*), showing structures compatible with spherical microglomeruli (19, 31, 35). The existence of bilobed HBs in the lateral protocerebrum of *Neohelice* has been previously suggested (33, 34). However, Golgi impregnations (Fig. 1*C*) unveiled the presence of another lobe, the vertical lobe, termed thus in correspondence to insect MBs (15). Consequently, the Golgi impregnations exposed HBs that closely resemble the calyxless MBs observed in some insects (15) (further discussion is provided in *SI Comment About Nomenclature*). The parallel fibers of gc constitute the pedunculus that bifurcates to give rise to the vertical lobe, medial lobe I, and medial lobe II (26, 31) (Fig. 1 *D* and *E*). The expression of p-CAMKII-α revealed also a view of the vertical and medial lobes, the HB small gc, and the pedunculus (Fig. 2*F*).

Neuronal elements previously described in crayfish HBs are parasol cells: approximately 200 neurons that are activated by olfactory, visual, and mechanical stimulation (26, 47). Parasol cells have been proposed as HBs' extrinsic neurons, resembling the output channel of MBs that receive multimodal sensory information from different Kenyon cells (19, 26). The intrinsic elements of the HBs shown in the present work appear not to resemble parasol cells. Unlike parasol cells that exclusively arborize within only one lobe of the HB, most of the fibers of gc arborize in the vertical lobe, the medial lobe I, and the medial lobe II (Fig. 1 *D* and *E*). In addition, big somata, as expected for parasol cells (26), are found neighboring the HB-gc cluster and do not present p-CaMKII-α immunoreactivity (Fig. 2*F*).

The Golgi impregnations also revealed claw-like specializations indicative of postsynaptic sites in the medial lobes (Fig. 1*F*) resembling both clawed Kenyon cells of MBs and intrinsic elements of HBs in hermit crabs (12, 15, 19). MBs' clawed Kenyon cells (also called class II or γ) are highly conserved across insect species and are proposed to represent an ancestral cell type (35). The presence of claw specializations in the medial lobes of *Neohelice* suggests input areas. Supporting the concept of multimodality of HBs, these lobes receive neuronal processes of the BLG2 neurons (Fig. 1*G*), which respond to mechanical and visual stimulation, integrate binocular visual information, and reduce their response to the VDS during training and testing sessions (44, 48). The interaction between these and intrinsic elements of HBs in the medial lobes may constitute a place where visual stimuli are integrated with other sensory modalities similar to what occurs in *Drosophila* MBs, where associative memories are processed in a centralized circuit that receives both visual and olfactory inputs (49–51).

Another concordance supporting homology is that adult neurogenesis occurs in both MBs and HBs (6, 38). The BrdU-positive labeling in the HB-gc cluster (Fig. 2*E*) is consistent with descriptions of adult neurogenesis in other crabs and in crayfish in a cluster that is called cluster A (20, 21, 31). Interestingly, a brain center that generates new neurons during adult life is just one of several similarities between the MBs and the hippocampus. In the vertebrate hippocampus, new granule cells add mossy fibers in parallel to the existing ensemble. In MBs and HBs, gc provide new parallel fibers to their neuropils (6, 20, 52). Several years after the seminal studies comparing MBs and vertebrate cortical structures on anatomical and physiological grounds (14), the remarkable work by Tomer et al. (7) added invaluable support for the hypotheses of a common origin of these higher brain centers. By the detailed comparison of the overall molecular topography of the brain anlage, the expression profile of several genes in forebrain-specific subregions during development, and the "molecular fingerprint" of vertebrate telencephalon and annelid MB neuron types, they showed that the vertebrate pallium and the annelid MBs most likely evolved from the same sensory associative brain center that was already present in their bilaterian ancestors (7). This conclusion is in accordance with the view that the ancient architecture of the paleo- and archipallium found in all vertebrates might have been present in the urbilaterian brain. The hippocampus's dentate gyrus, consisting of only one layer of principal neurons, noticeably resembles MBs: densely packed, basophilic somata of the gc that send out parallel processes intersected by afferent and efferent networks (14). Accordingly, recent studies by Wolff and Strausfeld (6, 12, 19) sustain that several anatomical items also support a genealogical correspondence. These items include, for instance, an extension from each intrinsic cell body of a long process always bearing presynaptic and postsynaptic specializations along its length, the intersection of the ensemble of such processes by sequential domains of afferent terminals and efferent dendrites forming an approximately orthogonal matrix, and the presence of recurrent interneurons within the ensemble. According to the authors' view, this ground pattern organization of HBs and MBs of arthropods is similar to the mammalian hippocampal structure, where the intrinsic cell bodies, the granule cell layer, send parallel axons that form an orthogonal matrix, interacting with local interneurons and afferent inputs from the entorhinal cortex, and projecting to the subiculum and back to the entorhinal cortex (6). Remarkably, in vertebrate archipallium structures, in MBs, and in HBs, several orthologous genes whose proteins play a critical role in the plasticity associated with memory processes (including p-CaMKII) show similar expression patterns (6). The present study tested the proposed similarity in functions (i.e., high-order memory processes) of MBs and HBs (13, 18, 25), a feature that adds functional evidence to the homology hypothesis (6, 22, 53).

**Neural Activity in the HBs.** Ex vivo recordings and neuroanatomical studies made in other decapods substantiate that the HBs have multimodal sensory inputs (25, 54). In the present study, we found that, like in medulla terminalis (45), calcium signals in the HBs are elicited by both mechanical and visual stimulation. In the medulla terminalis, proposed also to function as a region of higher order multimodal integration (25, 31, 32), we recently found that training induces specific changes in the neuronal responses triggered by the learned VDS (45); these changes correlate with memory persistence but not with long-term memory expression (55). The specific neuronal inputs to HBs that lead to the responses of both Mec and visual stimuli still need to be explored. The lobula giant neurons, which innervate the medial lobes (Fig. 1*G*) and respond to mechanical and visual inputs, are promissory candidates for this role (25, 43). Accordingly, and like in MBs of some insects, postsynaptic sites on the intrinsic cells of the medial lobes were found (12, 15, 19) (Fig. 1*F*).

Spontaneous and stimuli-triggered calcium signals in vertical and medial lobes were synchronized and highly correlated (Fig. S2). This finding was expected based on the Golgi impregnations showing the bifurcated structure of the intrinsic elements of the HB (Fig. 1 *C–E*) and from functional imaging studies on the axonal branches of the MB neurons (56). However, we also found sporadic spontaneous calcium signal transients that took place separately in both HB lobes (Fig. 4*C* and Fig. S2; arrows over traces in both figures). This observation might represent distinct inputs in the different lobes. Differential participation of the vertical and medial lobes in the activity elicited by different stimuli is also expected because it happens in the MBs (8, 50, 57).

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Either two-photon microscopy or a higher definition in the recording activity within each lobe would be necessary to shed light on this attribute of *Neohelice* HBs. In addition, HB activity presented slow oscillation components (Fig. S2). Oscillations have been described in both MBs of several insects and in HBs (47, 58). Interestingly, we did not observe such oscillations in the other protocerebral neuropil recently evaluated, the medulla terminalis (45).

**Memory-Related Neural Plasticity in the *Neohelice*'s HBs.** The HBs of crustaceans were long proposed to be centers of higher order integration that play a role in memory processes. The present study represents in vivo physiological evidence supporting that view (6, 19, 25, 26, 31). Here, we found that calcium signals triggered in the HB by the VDS decreased in both medial and vertical lobes during training. This decrease persisted at least until a short-term test 0.5 h after training. The decay of the CSR index was less pronounced in the vertical lobe than in the medial lobes, a difference that cannot be explained by disparity in the initial calcium signal to the training stimulus (all trained animals' VDS trial 1 summation $\%\Delta F/F$; mean $\pm$ SEM: medial lobes $46.38 \pm 21.21$, vertical lobe $41.12 \pm 17.60$; $t$ test for dependent samples: $t[12] = 0.77$, $P = 0.45$). The minor decay in the calcium signals of the vertical lobe could reflect, like in MBs (57), a differential involvement of the different input/output regions of the HB in this memory process.

The interpretation that the changes were induced by training rather than by a time-dependent decrease in the signals, such as modifications in dye distribution, photobleaching, or cytotoxicity, is supported by the fact that there were no changes between the responses elicited by the Mec before and after training. This view is also supported by the lack of changes in the UNs and by the recovery of the calcium signals elicited in response to the VDS when crabs were tested in a context different from the one used during training (Fig. 5). In addition, the changes in CSR are not likely explained by motion artifacts because escape responses are suppressed when the animals are held in the setup (44) and by the fact that the Mec triggered calcium signals but not escape responses (45).

MBs of insects are key structures for context-dependent memories. Their intrinsic neurons are required for visual memories, where their contribution to memory is proposed to involve linking items within a contextual framework (8, 15, 16, 51, 59). As previously mentioned, in *Neohelice,* the lobula giant neurons play a role in short- and long-term memory of the VDS stimulus; however, they show reduced responses to the learned VDS independent of a context-specific shift between training and testing (30). In this scenario, the HBs seem to be candidates for linking the VDS with the context, allowing memory expression when the VDS is presented in the learned context. In this study, we used a training protocol that yields context-specific memory expression in the short term (Fig. 3). Using the same training protocol for the calcium imaging experiments, we found that the $Ca^{2+}$ signal in response to the VDS was recovered in trained animals when the context was modified between training and testing (TR-dCTX; Figs. 4 and 5).

The present results constitute functional evidence in favor of a role of the HBs in memory processes that, similar to MBs (8, 15, 16), show neuronal changes correlated with a context-stimulus–specific association of the acquired information. These findings are in agreement with the historically proposed function for the HBs (25).

The hippocampus and MBs are key structures in processes that form long-term internal representations in their intrinsic neurons and categorize stimuli with respect to their indicative functions as cue and context (8, 9). The present study adds functional data to endorse the incorporation of crustacean HBs into this group, thus supporting the hypothesis for a common origin of high-order memory centers in bilateral animals (6, 7).

## Experimental Procedures

Crabs used were *N. granulata* (previously *C. granulata*) collected in the field and kept in the laboratory as described in *SI Materials and Methods*. The VDS used in behavioral and imaging experiments consisted of the displacement of a black rectangle passing above the animal in a 90° clockwise and anticlockwise excursion that was completed in 2.2 s. For in vivo calcium imaging experiments, a crystal of Calcium Green-1 dextran (Molecular Probes, Life Technologies) was stabbed in the neck of the pedunculus of the HB within the left eyestalk of the crab. Visual stimulation was done through the contralateral eye. The Mec consisted of pulses of nitrogen gas in the midright backside of the dorsal carapace. In dCTXs, the walls surrounding the animal were changed before testing. Imaging was done with a conventional epifluorescence microscope. Fluorescence data were analyzed with custom-written macros in Fiji/ImageJ (60). Research was conducted in accordance with the Ethical Reference Frame for Biomedical Investigations of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, equivalent to the standard procedures for animal care and use of the US NIH. Additional information is provided in *SI Materials and Methods*.

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